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# Changing Feeding Regimes To Demonstrate Flexible Biogas Production: Effects on Process Performance, Microbial Community Structure, and Methanogenesis Pathways

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**Flexible biogas production that adapts biogas output to energy demand can be regulated by changing feeding regimes. In this study, the effect of changes in feeding intervals on process performance, microbial community structure, and the methanogenesis pathway was investigated. Three different feeding regimes (once daily, every second day, and every 2 h) at the same organic loading rate were studied in continuously stirred tank reactors treating distiller's dried grains with solubles. A larger amount of biogas was produced after feeding in the reactors fed less frequently (once per day and every second day), whereas the amount remained constant in the reactor fed more frequently (every 2 h), indicating the suitability of the former for the flexible production of biogas. Compared to the conventional more frequent feeding regimes, a methane yield that was up to 14% higher and an improved stability of the process against organic overloading were achieved by employing less frequent feeding regimes. The community structures of bacteria and methanogenic archaea were monitored by terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA and *mcrA* genes, respectively. The results showed that the composition of the bacterial community varied under the different feeding regimes, and the observed T-RFLP patterns were best explained by the differences in the total ammonia nitrogen concentrations, $H_2$ levels, and pH values. However, the methanogenic community remained stable under all feeding regimes, with the dominance of the *Methanosarcina* genus followed by that of the *Methanobacterium* genus. Stable isotope analysis showed that the average amount of methane produced during each feeding event by acetoclastic and hydrogenotrophic methanogenesis was not influenced by the three different feeding regimes.**

Interest in a demand-driven biogas supply for flexible electricity production with the aim of balancing the supply of electricity generated from sources producing fluctuating amounts of electricity, such as solar and wind sources, has increased recently. Different strategies can be employed to obtain a demand-driven biogas supply, including a strategy involving a conventional biogas plant with biogas storage or a strategy involving a conventional biogas plant with a biogas upgrade to biomethane for subsequent storage in a natural gas grid (1–3). Conventional biogas production with integrated heat and power (CHP) plants are normally run on a semicontinuous substrate feeding regime in order to provide a constant biogas output and electricity generation (4).

Alternatively, flexible biogas production that adapts biogas output to energy demand can be implemented by feeding management, including varying the feeding regimes and substrate composition. The production of larger amounts of biogas can be achieved immediately after feeding, and smaller amounts of biogas production are achieved during the nonfeeding period. Compared to the conventional operation of biogas plants with biogas storage, flexible biogas production profits from a reduced need for gas storage due to the production of large and small amounts of biogas at the times of high and low energy demand, respectively (2, 4). However, additional CHP plant capacity is required to meet the need for a high biogas production rate after feeding at the times of high energy demand. Therefore, the overall economy of flexible biogas production depends on whether the additional income gain is able to cover the additional investment and operational costs, if any (3–5).

The concept of flexible biogas production has recently been introduced, and only a few studies have looked at the effects of feeding management on process performance, microbial community structure, and methanogenesis pathways. More frequent feeding is usually preferred in the conventional operation of biogas plants, because in common practice it is believed to help the stability of the process (6). A change from more to less frequent feeding at the same organic loading rate (OLR) may lead to process disturbance, such as volatile fatty acid (VFA) accumulation, a lower pH, and a mismatch between the growth rates of VFA-producing and -consuming microbes (7, 8). Lv et al. compared the effects of two feeding regimes (once per day and twice per day) with the same daily organic load on process performance and methanogenesis pathways in maize silage-fed continuously stirred

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tank reactors (CSTRs) (9). The reactor fed once daily showed that VFAs transiently accumulated with a concomitant reduction in pH after feeding but returned to normal levels before the next feeding event. Stable isotope analysis of the gases produced demonstrated a temporal variation in methanogenesis which correlated with the change in the VFA concentration. The reactor that was fed twice per day was shown to be less dynamic with a constant methane production rate, VFA concentration, pH, and level of methanogenesis after feeding. Similar findings were reported by Mauky et al. (10) for CSTRs fed with maize silage, sugar beet silage, and cattle slurry, where less frequent feeding led to alteration of the methane, carbon dioxide, hydrogen, and hydrogen sulfide contents in biogas, pH level, and VFA concentration without VFA accumulation over a long operating period of 200 days. Another study of acetate-fed reactors demonstrated that reactors fed hourly and daily were dominated by *Methanosaeta* and *Methanosarcina*, respectively, with the latter being even more tolerant to environmental perturbation (organic overloading) (11). Another study based on synthetic raw domestic sewage demonstrated that a reactor fed every second day had a higher tolerance to an organic shock load of 8 g of chemical oxygen demand liter$^{-1}$ and high total ammonia nitrogen (TAN) levels of up to 8,000 mg $NH_4^+$-N liter$^{-1}$ than the reactor fed daily, which was in part due to the higher degree of bacterial dynamics in the former (12). Despite these previous studies, our understanding of the microbial community structure, methanogenesis, and biogas production process performance for reactors fed at different feeding intervals is still limited. Moreover, the findings from the reactors fed with acetate (11) and synthetic feed materials (12) cannot be easily translated to typical biogas plants working with complex agricultural and organic industrial wastes.

In this study, two laboratory-scale CSTRs were fed with distiller's dried grains with solubles (DDGS) under identical operating conditions except for the use of different feeding intervals (every 2 h, once a day, and every second day). The more frequent feeding (every 2 h) mimics the frequency of feeding in conventional biogas plants that run on a semicontinuous substrate feeding mode to achieve nearly the same amount of biogas and electricity generation throughout the day. The less frequent feeding interval (once a day and every second day) assumes flexible biogas production with the aim of high biogas production rates for a few hours after feeding and lower biogas production rates during the extended nonfeeding period. A longer feeding interval over a period of 2 days, which mimics a typical low-energy-demand period on a weekend, was studied.

DDGS is a by-product of bioethanol production plants. It is produced from a fermentation waste also known as stillage after passage through centrifugation and drying stages. Due to the reduction in water content, DDGS can easily be transported to a place where it is used as animal feed. Since DDGS drying accounts for approximately 30% of energy consumption of a bioethanol production plant (13), the use of stillage prior to drying for biogas production has environmental and economic benefits, especially where both plants are located in close proximity to each other (14, 15).

The aim of this study was to investigate flexible biogas production by changing feeding intervals and the assessment of its effect on process performance, microbial community structure, and the methanogenesis pathway of DDGS-fed CSTRs under mesophilic conditions. The community structures of bacteria and methanogenic archaea were monitored by terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA and *mcrA* genes, respectively. The carbon isotope signatures of methane and carbon dioxide were analyzed to quantify hydrogenotrophic methanogenesis (HM) and acetoclastic methanogenesis (AM). Any correlations between the methanogenesis pathways studied by isotope analysis and the methanogenic community structure studied by molecular methods were further assessed. Moreover, a short-term stress condition was initiated by increasing the OLR in reactors fed at different intervals in order to evaluate the level of tolerance to environmental perturbation.

## MATERIALS AND METHODS

**Substrate.** All biogas reactors were fed with DDGS and were supplemented with FerroSorp DG (HeGo, Biotec, Germany) and trace element solution (TES). DDGS was obtained from an industrial-scale bioethanol plant (CropEnergies AG, Zeitz, Germany) where wheat is the main raw material. FerroSorp is a powdery substance containing iron hydroxide that was added to the reactors to precipitate hydrogen sulfide. The TES contains the following (all concentrations are in grams liter$^{-1}$): Ni(II)·$6H_2O$, 2.13; Co(II)·$6H_2O$, 0.531; $NaMoO_4$·$2H_2O$, 0.332; $(NH_4)_6H_2W_{12}O_{40}$·$xH_2O$, 0.423. The daily substrate was prepared by mixing 66.34 g DDGS, 2.0 ml TES, and 2.56 g FerroSorp DG, and finally, water was added to the mixture to make a total of 380 ml of solution.

**Reactor setup.** Two laboratory-scale CSTRs were operated under mesophilic conditions (38°C) with DDGS as the only substrate. The total volume of each reactor was 15 liters, and the working volume was 10 liters. Before the start of this study, the two reactors were operated under identical conditions, and then the contents of the reactors were mixed together to set up equivalent starting conditions for this study. Three different feeding regimes were studied: once daily, every second day, and every 2 h. Except for the difference in feeding regimes, the two reactors were operated under identical operating conditions, including the same OLR and hydraulic retention time (HRT). DDGS was fed manually in the regimes with feeding daily and every second day, whereas a peristaltic pump was used in the regime with feeding every 2 h. The whole experiment was divided into 4 phases (Table 1): (i) both reactors A and B were fed once daily (phase I, days 1 to 29), (ii) reactor A was fed once daily and reactor B was fed every 2 h (phase II, days 30 to 63), (iii) reactor A was fed every second day and reactor B was fed every 2 h (phase III, days 64 to 107), and (iv) reactor A was fed every second day and reactor B was fed every 2 h but at an OLR higher than that in phase III (phase IV, days 108 to 118). The feeding regime in phases I to III was changed after the process reached a steady state. We defined the steady state to be the point when we observed biogas production rates within 10% of their average values between feeding events after an operating period of at least one HRT period (Table 1) (16, 17). In phases I to III, the OLR and HRT of both reactors were 4 g volatile solids (VS) liter$^{-1}$ day$^{-1}$ and 26 days, respectively. In phase IV, a short-term stress condition was employed by gradually increasing the OLR from 4 to 11 g VS liter$^{-1}$ day$^{-1}$ within the last 10 days of the operating period. In phase IV, the HRT was 26 days for an OLR that ranged from 5 to 7 g VS liter$^{-1}$ day$^{-1}$, whereas the HRT was shortened to 14 and 10 days at OLRs of 9 and 11 g VS liter$^{-1}$ day$^{-1}$, respectively. Due to the difficulty with the pumping of the feed at OLRs of 9 and 11 g VS liter$^{-1}$ day$^{-1}$ in a total feed volume of 380 ml (a larger amount of total solids than that with feed at a lower OLR), the feed was further diluted with water so that the total daily feed volumes were 700 and 1,000 ml, respectively, and as a consequence, the HRT was shortened to 14 and 10 days, respectively.

**Basic process parameters.** The volume of biogas was automatically recorded by a gas meter (TG 0.5; Dr.-Ing. Ritter Apparatebau GmbH & Co. KG, Bochum, Germany). The volume of the biogas was corrected to standard temperature (273.15 K) and pressure (101.325 kPa) and is reported as normalized liters ($liters_N$). The biogas that vented from the gas meter was collected in a gas bag, and once the gas bag was full, all the

TABLE 1 DDGS-fed reactor operating conditions and process parameters[a]

| Phase (days of study) | Reactor | OLR (g VS liter$^{-1}$ day$^{-1}$) | Feeding mode | Total $CH_4$ production (liters$_N$ day$^{-1}$)[b] | Total biogas production (liters$_N$ day$^{-1}$)[b] | SMP (ml$_N$ g VS$^{-1}$)[b] | SBP (ml$_N$ g VS$^{-1}$)[b] | $CH_4$ (%) | $H_2$ concn (ppm) | pH | VOA concn (g liter$^{-1}$) | VOA/TIC ratio (g VOA g $CaCO_3^{-1}$) | TAN concn (g $NH_4^+$-N liter$^{-1}$) | Acetate concn (mg liter$^{-1}$) | VFA concn (mg HAc eq liter$^{-1}$) | VSR (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I (1–29) | A | 4 | Daily | 16.1 ± 0.8 | 28.2 ± 1.5 | 402 ± 19.3 | 710 ± 38.7 | 57.2 ± 0.5 | 59.4 ± 9.2 | 7.82 ± 0.05 | 1.28 ± 0.04 | 0.14 ± 0.01 | 3.5 ± 0.1 | 57.0 ± 3.8 | 65.8 ± 3.4 | 61.7 ± 0.3 |
| | B | 4 | Daily | 16.2 ± 0.6 | 28.2 ± 1.2 | 400 ± 14.3 | 710 ± 29.4 | 57.4 ± 0.6 | 68.3 ± 13.2 | 7.86 ± 0.05 | 1.26 ± 0.01 | 0.13 ± 0.00 | 3.6 ± 0.1 | 55.5 ± 4.8 | 65.5 ± 5.3 | 62.7 ± 0.5 |
| II (30–63) | A | 4 | Daily | 16.5 ± 0.4 | 28.7 ± 0.7 | 414 ± 10.5 | 724 ± 17.4 | 57.3 ± 0.3 | 61.1 ± 6.7 | 7.81 ± 0.02 | 1.07 ± 0.08 | 0.13 ± 0.01 | 3.1 ± 0.3 | 39.3 ± 3.0 | 57.7 ± 5.3 | 61.3 ± 0.1 |
| | B | 4 | Every 2 h | 14.4 ± 0.6 | 24.7 ± 1.1 | 363 ± 15.7 | 622 ± 27.0 | 58.4 ± 0.4 | 31.7 ± 10.6 | 7.78 ± 0.04 | 1.09 ± 0.06 | 0.13 ± 0.00 | 2.9 ± 0.01 | 42.9 ± 1.8 | 58.4 ± 2.5 | 59.4 ± 0.4 |
| III (64–107) | A | 4 | Every second day | 15.9 ± 0.6 | 28.7 ± 1.2 | 402 ± 15.6 | 724 ± 31.1 | 58.2 ± 0.3 | 41.4 ± 8.2 | 7.78 ± 0.02 | 1.12 ± 0.11 | 0.12 ± 0.01 | 3.1 ± 0.2 | 43.0 ± 3.1 | 52.2 ± 3.1 | 62.1 ± 0.3 |
| | B | 4 | Every 2 h | 14.1 ± 0.6 | 24.3 ± 1.1 | 354 ± 16.3 | 613 ± 27.3 | 57.8 ± 0.4 | 8.7 ± 3.1 | 7.71 ± 0.01 | 1.06 ± 0.06 | 0.13 ± 0.01 | 2.6 ± 0.1 | 27.8 ± 2.0 | 35.0 ± 4.3 | 58.6 ± 0.5 |
| IV (108–118) | A | 5–11 | Every second day | 29.2 | 51.3 | 387 | 689 | 59.8 | 75.4 | 7.76 | 1.25 | 0.13 | 3.3 | 54.2 | 62.3 | NM |
| | B | 5–11 | Every 2 h | 23.0 | 39.6 | 304 | 526 | 57.5 | 20.4 | 7.67 | 1.17 | 0.13 | 2.7 | 44.9 | 55.6 | NM |

[a] The process parameters in phases I to III are the average values after steady state was achieved. Process parameters in phase IV are the average values for a 10-day operating period under stress conditions (when OLR was increased from 5 to 11 g VS liter$^{-1}$ day$^{-1}$). OLR, organic loading rate; VS, volatile solid; VSR, volatile solid reduction; SMP, specific methane production; SBP, specific biogas production; VOA, volatile organic acids; TIC, total inorganic carbon; TAN, total ammonia nitrogen; VFA, volatile fatty acids; HAc eq, acetic acid equivalent of all VFAs; NM, not measured.

[b] Data were corrected to standard temperature (273.15 K) and pressure (101.325 kPa) and are reported as normalized liters (liters$_N$) or normalized milliliters (ml$_N$).

biogas was transferred into an AwiFlex gas analyzer (Awite Bioenergie GmbH, Germany) for measurement of the composition of the biogas. The AwiFlex gas analyzer was equipped with optical infrared sensors to determine the concentrations of $CH_4$ and $CO_2$ as well as with electrochemical sensors to determine the concentrations of $O_2$, $H_2$, and $H_2S$ (detection limit, 5,000 ppm).

Liquid effluent was periodically collected from both reactors at the same time point, and its pH was measured immediately. It was then centrifuged at 20,000 × *g* and 10°C for 12 min, and the supernatant solution was filtered through a sieve with a mesh size of 1 mm. The supernatant solution was used for further analysis of VFA, volatile organic acids (VOA), and total inorganic carbonate (TIC) as well as TAN, expressed in grams of $NH_4^+$-N liter$^{-1}$ (i.e., TAN is defined as a sum of the amount of free ammonia nitrogen [FAN] plus the amount of ammonium nitrogen). For VFA analysis, an aliquot (5 ml) of the supernatant solution was transferred into a 20-ml vial followed by the addition of 1 ml internal standard (2-ethylbutyric acid, 180 mg liter$^{-1}$) and 1 ml phosphoric acid (50% [vol/vol]). The vial was immediately sealed with a butyl rubber stopper and an aluminum crimp. The concentration of VFAs was determined from the headspace by use of a 5890 series II gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) as described earlier (18). For TAN analysis, an aliquot (125 μl) of the solution was diluted to 1:2,000 with distilled water and the TAN was determined by a standard Nessler method using a benchtop spectrometer (DR 3900; Hach-Lange, Loveland, CO, USA). VOA and TIC were determined using a Titration Excellence T 90 titrator (Mettler-Toledo, Switzerland) as described previously (18). Total solids (TS) and VS were analyzed according to a standard method (19). The VS reduction (VSR) was calculated on the basis of the number of grams of VS of the feed and effluent according to the following equation: VSR = [1 − (number of grams of VS of effluent/number of grams of VS of feed)] × 100%. The specific methane production (SMP) and specific biogas production (SBP) were calculated on the basis of the daily average amounts of methane produced (normalized milliliters day$^{-1}$) and biogas produced (normalized milliliters day$^{-1}$) divided by the daily average amount of substrate fed on a VS basis (number of grams of VS day$^{-1}$), respectively. SMP and SBP represent the methane and biogas yields, respectively.

**Stable isotope analysis.** Biogas samples were periodically collected from the headspace of the reactor in triplicate and stored in a gastight evacuated vial (20 ml) until further analysis. The stable isotope analysis of $^{13}C/^{12}C$ gas samples was performed using a gas chromatography (GC)-combustion-isotope ratio mass spectrometry (IRMS) system consisting of a gas chromatograph (HP 6890 series; Agilent Technology, Santa Clara, CA, USA) coupled with an IRMS (Finnigan MAT 253; Thermo Finnigan, Bremen, Germany) via a combustion interface. For the GC separation of $CH_4$ and $CO_2$, gas samples (30 μl) were injected manually into the GC instrument equipped with a CP-Porabond Q column (50 m by 0.32 mm by 0.5 μm; Varian, USA). The column temperature was kept constant at 40°C, and the flow rate of helium was 2 ml/min.

The stable carbon isotope data were reported in delta notation ($\delta^{13}C$) in units of parts per thousand (‰) versus the Vienna Pee Dee Belemnite (V-PDB) standard: $\delta^{13}C = \{[(R_a)\text{sample}/(R_a)\text{standard}] - 1\} \times 10^3$ (in parts per thousand), where $R_a$ is the $^{13}C/^{12}C$ ratio (20). The relationship among the $^{13}C$ isotopic signatures of total $CH_4$ ($\delta^{13}CH_4$), acetate-derived $CH_4$ ($\delta_{ma}$), and $CO_2$-derived $CH_4$ ($\delta_{mc}$) can be described using the following mass balance equation (21):

$$\delta^{13}CH_4 = f_{mc} \cdot \delta_{mc} + (1 - f_{mc}) \cdot \delta_{ma} \quad (1)$$

where $f_{mc}$ is the fraction of $CH_4$ produced from the reduction of $CO_2$ via HM.

To estimate $f_{mc}$, the values of $\delta_{ma}$ and $\delta_{mc}$ need to be known beforehand. Therefore, an additional batch experiment was conducted using inoculum obtained from both CSTRs (A and B) in phase III on day 72 (see the supplemental material). Fluoromethane ($CH_3F$) was added into the batch bottles to inhibit AM. Controls without inhibitor were incubated in parallel with $CH_3F$-supplemented batch bottles. Any methane production in the $CH_3F$-supplemented batch bottles (AM inhibited) is therefore due

to HM and was used to estimate the value of $\delta_{mc}$. The $\delta^{13}CH_4$ in the bottles in which AM was inhibited was, on average, −68.5‰ (data not reported), and thus, $\delta_{mc}$ was equal to −68.5‰. Then, $\delta_{ma}$ was estimated using equation 2:

$$\delta_{ma} = \delta_{ac} + \varepsilon_{ma} \quad (2)$$

where $\delta_{ac}$ is the $^{13}C$ isotopic signature of acetate and $\varepsilon_{ma}$ is the level of isotopic enrichment by AM. In pure culture, when substrate is not limiting, the isotope fractionation factor for AM alone can reach up to 1.027 (equivalently, an isotope enrichment factor [$\varepsilon_{ma}$] of −27‰) for the genus *Methanosarcina* (22), members of which comprised the dominant acetoclastic methanogens in all of our DDGS-fed reactors (see Fig. 3). Because the $\varepsilon_{ma}$ value is affected by the concentration of acetate (22) and because the concentration of acetate in all of the DDGS-fed reactors was generally low (less than 365 mg liter$^{-1}$) compared to the concentration reported (1,200 mg liter$^{-1}$) in the previous study (22), we assumed a low fractionation factor ($\varepsilon_{ma}$ = −10‰). Because the carbon isotope fractionation between the biomass ($\delta^{13}$ of DDGS = −22.5‰) and the fermentatively produced acetate is negligible (21) and because the $\delta^{13}$ of biomass may be used as a proxy for mass balance calculations (23), we assumed the same value for the $^{13}C$ isotopic signature of acetate ($\delta_{ac}$) and the biomass (i.e., $\delta_{ac}$ = −22.5‰). Using this assumption and equation 2, the value of $\delta_{ma}$ was estimated to be −32.5‰. This value is more realistic since the $\delta^{13}CH_4$ value of −41‰ was observed in the control batch incubation with DDGS as a monosubstrate (data not reported). If we assume a high isotope fractionation factor (for instance, $\varepsilon_{ma}$ = −21‰), then the calculated $\delta_{ma}$ value is −43.5‰, which is different from the observed $\delta^{13}CH_4$ value of −41‰. Considering the level of methane production through both pathways and the observed $\delta^{13}CH_4$ value of −41‰ in the control batch incubation, the fractionation factor should be less than 1.018 and our assumption that $\varepsilon_{ma}$ is equal to −10‰ is therefore realistic. To summarize, the fractions of hydrogenotrophic methanogenesis to methane production in the CSTRs were estimated according to equation 1 using the measured values of $\delta^{13}CH_4$ in the CSTRs, and $\delta_{mc}$ was equal to −68.5‰ and $\delta_{ma}$ was equal to −32.5‰.

**DNA extraction and purification.** Samples for microbial community analyses were taken right before substrate feeding during all experimental phases (phases I to IV). During each phase, liquid samples were collected at two different sampling points in phases I to III (at about 2 weeks after the beginning of each phase and right before the start of the next phase, i.e., days 16 and 29, respectively, in phase I; days 37 and 59, respectively, in phase II; days 81 and 107, respectively, in phase III) and at one sampling point in phase IV (i.e., day 115). Samples were collected in sterile Eppendorf tubes and stored in a freezer (−80°C) until further analysis. DNA was isolated in duplicate from 400 mg of the sample with a NucleoSpin soil kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions, and its quality was checked by 1.5% agarose gel electrophoresis and quantified with a NanoDrop ND-1000 UV/visible spectral photometer (PeqLab, Germany).

**PCR.** Bacterial 16S rRNA gene fragments were amplified by PCR using universal bacterial primers 27F (5′-AGAGTTTGGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) as described earlier (18), whereas *mcrA* genes were amplified by using the *mcrA*- and *mrtA*-specific forward primer mlas and the reverse primer mcrA-rev as described earlier (24). For T-RFLP analysis, the same primers used for PCR amplification were used, except that the reverse primer was 5′ labeled with the phosphoramidite fluorochrome 6-carboxyfluorescein. PCR products were checked by 1.5% gel electrophoresis. Then, the PCR products were purified using a SureCleanPlus kit (Bioline, Germany) and quantified using the NanoDrop ND-1000 UV/visible spectral photometer (PeqLab, Germany).

**T-RFLP analysis based on 16S rRNA and *mcrA* genes.** T-RFLP analysis was performed as described before (24). Briefly, bacterial 16S rRNA genes were subjected to restriction enzyme digestion with either the restriction endonuclease HaeIII or the restriction endonuclease MspI (New England BioLabs, Schwalbach, Germany), whereas *mcrA* genes were digested with BstNI (New England BioLabs, Schwalbach, Germany). After the purification of the terminal restriction fragments (T-RFs), they were resuspended in HiDi formamide containing a GeneScan-500 carboxy-X-rhodamine standard (Applied Biosystems, Weiterstadt, Germany) and a MapMarker 1000 standard (Bioventures Inc., Murfreesboro, TN, USA) for *mcrA* and bacterial 16S rRNA gene analyses, respectively. Fluorescently labeled T-RFs were separated using capillary electrophoresis with an automatic sequencer (ABI Prism 3130xl genetic analyzer; Applied Biosystems, Weiterstadt, Germany). T-RFLP data were compared with the data for the internal standards using GeneMapper (version 3.7) software (Applied Biosystems, Weiterstadt, Germany) and were exported to R script, peak areas were normalized, and a noise filtering ($\sigma$ = 5 [i.e., signals with low intensity were removed according to five times the standard deviation of the data set]) was applied. T-RFs in the size ranges of 50 to 500 and 50 to 1,000 bp were considered for subsequent analysis of the archaeal *mcrA* and bacterial 16S rRNA genes, respectively. For statistical analysis, T-RFLP data sets were reduced by removing T-RFs with a low abundance (below 1%). Relative T-RF abundances were calculated by dividing the peak area for reach T-RF by the total peak area. Linking of taxonomic information to the major *mcrA* T-RFs was done by using the sequence and T-RFLP database from previous studies by our group (9, 24). An example of the T-RFLP profiles of duplicate samples is provided in order to show the reproducibility of the T-RFLP fingerprinting approach (see Fig. S1 in the supplemental material).

**Statistical analysis.** Multivariate statistical analysis based on the vegan package of R (version 3.0.1) (25) was performed on the T-RFLP profiles in order to determine the variability of the microbial communities. Bray-Curtis dissimilarity indices, which include relative abundance information, in addition to the presence and absence of T-RFs, were applied. The multidimensional dissimilarity matrix was reduced to two dimensions showing the dissimilarities between the community structures of samples as distances on the plot. The major process parameters correlating with community composition were fitted using the envfit algorithm. The significance of single process parameters on the nonmetric multidimensional scaling (nMDS) ordination results was tested using a Monte Carlo test with 1,000 permutations, and only significant parameters ($P < 0.05$) are shown.

The statistical significances of the differences in the main process parameters between the two reactors were determined using the Student *t* test. The level of accepted statistical significance was a *P* value of <0.05.

## RESULTS

**Process performance.** The effect of a change in feeding regimes (feeding every 2 h, once daily, and every second day) on process performance was studied in laboratory-scale CSTRs fed with DDGS. The basic process parameters and isotope signatures of $CH_4$ and $CO_2$ were monitored over 128 days during four experimental phases (Fig. 1). During phase I, reactors A and B were operated under identical conditions (they were fed once daily, and OLR was 4 g VS liter$^{-1}$ day$^{-1}$) to test the homogeneity of the two reactors before proceeding to the next phase, where different feeding regimes were employed. During phases II to IV, reactors A and B were operated under identical conditions, except the feeding intervals differed. The reactor characteristics and the steady-state data are summarized in Table 1. Since reactors A and B were operated under identical conditions with daily feeding during phase I, there were no significant differences in process performance between the two reactors ($P > 0.05$; Table 1). This result demonstrates that the process performances of both reactors were almost identical before any change in feeding intervals was employed in subsequent experimental phases.

Since reactor A was fed daily in phase II, as in phase I, process performances were almost the same in the two phases (average

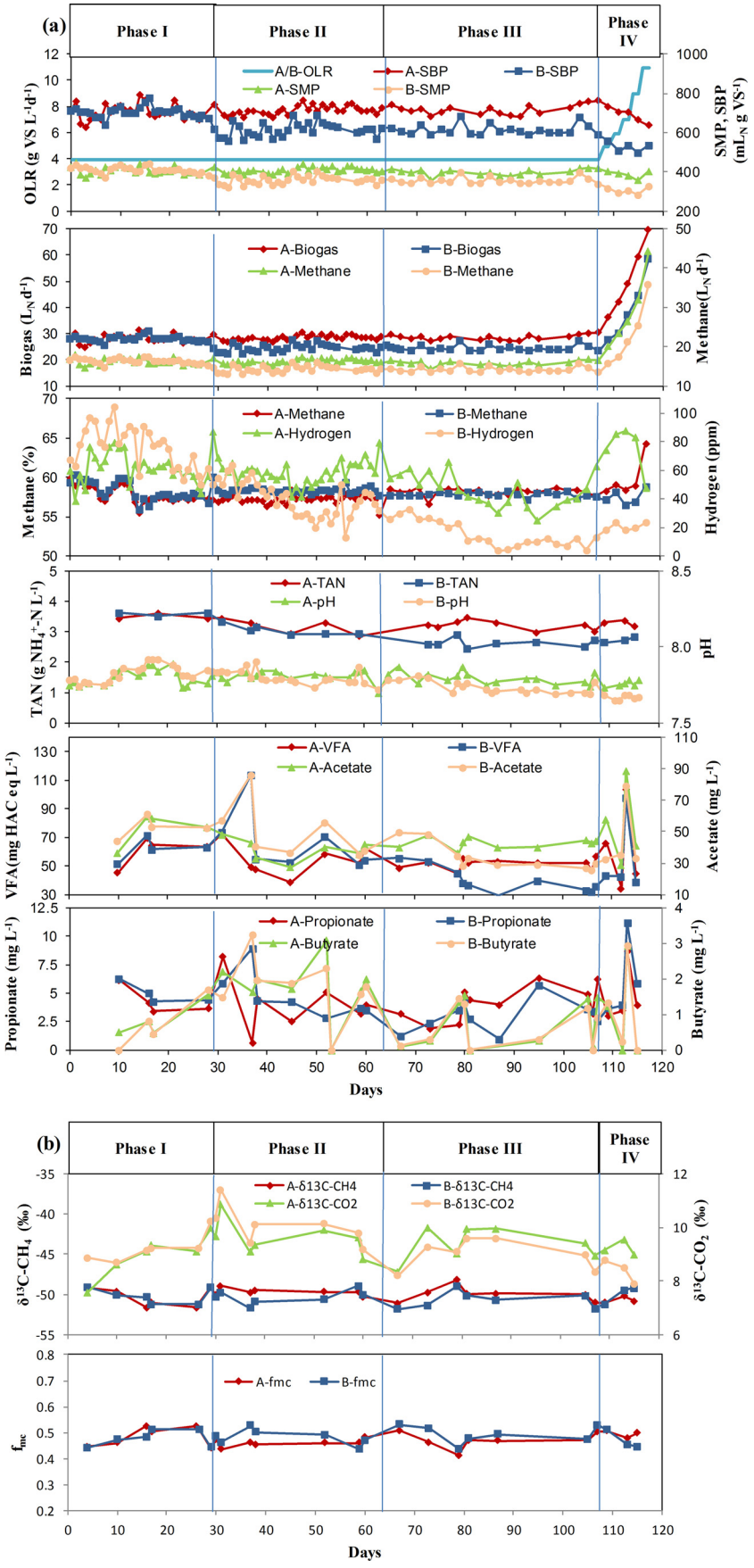


FIG 1 Long-term process parameters (a) and isotope signatures of $CH_4$ and $CO_2$ (b) of DDGS-fed reactors monitored over phases I to IV. The value for butyrate is the sum of the concentrations of *n*-butyrate and isobutyrate. $f_{mc}$ is the fraction of methane produced through hydrogenotrophic methanogenesis. For butyrate data, values of 0 indicate that the values were below the detection limit.

biogas and methane production in phase II, 28.7 ± 0.7 and 16.5 ± 0.4 liters$_N$ day$^{-1}$, respectively; SMP and SBP in phase II, 414 ± 10.5 and 724 ± 17.4 ml$_N$ g VS$^{-1}$, respectively). However, reactor B fed every 2 h showed a reduced process performance (biogas production, $CH_4$ production, SMP, and SBP, 24.7 ± 1.1 liters$_N$ day$^{-1}$, 14.4 ± 0.6 liters$_N$ day$^{-1}$, 363 ± 15.7 ml$_N$ g VS$^{-1}$, and 622 ± 27.0 ml$_N$ g VS$^{-1}$, respectively). The process efficiency deteriorated considerably when the feeding was changed from daily to every 2 h in reactor B. Compared to the yields obtained with daily feeding, methane and biogas yields were significantly lower by 14 and 16%, respectively, in the reactor fed every 2 h ($P < 0.05$). The VSRs were 61.3% ± 0.1% and 59.4% ± 0.4% for reactors A and B, respectively (Table 1), showing statistically significant differences in the degradation of organic matter between the reactors fed daily and those fed every 2 h. Interestingly, there was no significant difference between reactors A and B regarding other process parameters [VOA concentration, 1.1 g liter$^{-1}$; VOA/TIC, about 0.13 g VOA g $CaCO_3^{-1}$; TAN concentration, about 3 g $NH_4^+$-N liter$^{-1}$; acetate concentration, 39 to 43 mg liter$^{-1}$; total VFA concentration, about 58 mg acetic acid (HAc) eq liter$^{-1}$]. The average daily biogas composition remained almost the same in both reactors ($CH_4$, about 58%; $CO_2$, 42%; $H_2$, 32 to 61 ppm). However, the biogas composition and the concentration of VFA varied a lot between two feeding events in the reactor fed daily, whereas they remained constant in the reactor fed every 2 h (see Discussion).

In phase III, the feeding interval was changed from once daily to every second day in reactor A, whereas the feeding interval of reactor B was kept the same at an interval of every 2 h. The process efficiencies (SBP and SMP) were almost the same in the reactors fed daily (phase II) and every second day (phase III) (Table 1). On the other hand, SBP and SMP were significantly higher by about 18 and 13%, respectively ($P < 0.05$), in the reactor fed every second day than in the reactor fed every 2 h. The VSR as well as the concentrations of TAN, $H_2$, acetate, and total VFAs were significantly higher in the reactor fed every second day. The higher TAN level in the reactors fed every second day implies that the protein fraction of the DDGS was better hydrolyzed and fermented in subsequent steps. However, free ammonia did not reach an inhibitory level. The higher VSR indicates the better degradation of the organic matter in the reactor fed every second day as well. Despite the significant difference between the concentrations of total VFAs and acetate in both reactors, the residual acetate and VFA concentrations were low (acetate concentrations, 28 to 43 mg liter$^{-1}$; total VFA concentrations, 35 to 52 mg HAc eq liter$^{-1}$). This shows, irrespective of the feeding intervals, that the VFA production rate by fermentative bacteria and the subsequent production of acetate by acetogenic bacteria were well balanced with the acetate consumption rate through the AM and/or syntrophic acetate oxidation (SAO) pathway. The pH ranged from 7.69 to 7.87 under all feeding regimes, which is optimal for a biogas production process (26, 27).

In phase IV, a short-term stress test was conducted by gradually increasing the OLR from 4 to 11 g VS liter$^{-1}$ day$^{-1}$ during the last 10 days of the operating period (Fig. 1). The level of biogas production increased significantly in both reactors with an increase in the OLR. However, an increase in the OLR led to a reduction of SBP and SMP in both reactors. Compared to the SMP at steady state in phase III, the reduction in SMP under stress conditions was only 3.6% and was greater at about 14% in the reactors fed every second day and every 2 h, respectively. Similar to SMP, SBP was reduced by only 4.8% and about 14% in the reactors fed every second day and every 2 h, respectively. This indicates that despite the reduction in SMP and SBP in both reactors under the stress condition, the reactor fed every second day still performed much better than the reactor fed every 2 h. Nevertheless, both reactors maintained an optimum pH range and a low residual VFA concentration under the stress condition.

In addition to the long-term process parameters presented above, short-term process parameters were also monitored over a period of 48 h in phase III (Fig. 2). Biogas production was highly dynamic in reactor A fed every second day, whereby the highest biogas production rate (4.6 liters$_N$ h$^{-1}$) was observed within 1 h after feeding and the lowest rate was 0.5 liters$_N$ h$^{-1}$ right before the next feeding event (Fig. 2a). The biogas production rate in this reactor during the first 24 h was always higher than that in the reactor fed every 2 h. The methane content in this reactor was reduced to a minimum value of 47% within 4 h after feeding and finally returned to the initial value of 60% after 12 h and remained at 60% until the next feeding event (Fig. 2c). The gas quality was assessed at a frequency of up to every 2 h. That means that each measurement results from the average gas quality collected over at least 2 h, and thus, real-time gas quality was even more volatile than the measurement data can show. The quality of the biogas in terms of combustibility was lower within 4 h after feeding because of the lower methane content in the biogas. However, biogas is normally stored for several hours during the operation of a full-scale biogas plant. This allows biogas with different compositions to be mixed in the storage tank or the headspace before it is used for electricity generation in CHP units (10). Therefore, the quality of the biogas that reaches the CHP unit is more stable and better than that of the biogas generated within 4 h after feeding. The methane produced within one feeding event (48 h) in the reactor fed every second day had an average methane content of 58 to 60% of that of the reactor fed every 2 h (Table 1). The concentrations of $H_2$ and total VFAs in the reactor fed every second day increased significantly after feeding and reached peak values of 127 ppm at 2.5 h and 664 mg HAc eq liter$^{-1}$ at 7 h, respectively (Fig. 2c and d). Both $H_2$ and VFA returned to their normal values during the nonfeeding period. The concentrations of individual VFAs, such as acetate, propionate, and butyrate, fluctuated over the uncritical ranges of 30 to 343, 2 to 278, and 0.2 to 11.5 mg liter$^{-1}$, respectively (Fig. 2d and e). A significant increase in $H_2$ and VFA concentrations after feeding in the reactor fed every second day indicates the activity of the hydrolysis, acidogenesis, and acetogenesis steps. On the other hand, the biogas production process was less dynamic in reactor B fed every 2 h, with a narrow biogas production rate range (0.8 to 1.6 liters$_N$ h$^{-1}$), an almost constant $CH_4$ content (59%), a narrow range of concentrations of total and individual VFAs (total VFA concentration range, 21 to 56 mg HAc eq liter$^{-1}$; acetate concentration range, 15 to 41 mg liter$^{-1}$; propionate concentration range, 2.2 to 5.6 mg liter$^{-1}$; butyrate concentration range, <1.8 mg liter$^{-1}$) and lower $H_2$ concentrations (range, 7 to 40 ppm). Moreover, in phases I and II, the reactor fed daily was also more dynamic with regard to biogas and VFA production than the reactor fed every 2 h (data not shown).

**Stable isotope signatures and methanogenic pathways.** The carbon isotope signatures of $CO_2$ and $CH_4$ over the 118-day operating period were obtained for the gas samples collected right before feeding (Fig. 1b). $\delta^{13}CH_4$ values can be used to differentiate between AM and HM pathways, with values showing greater de-

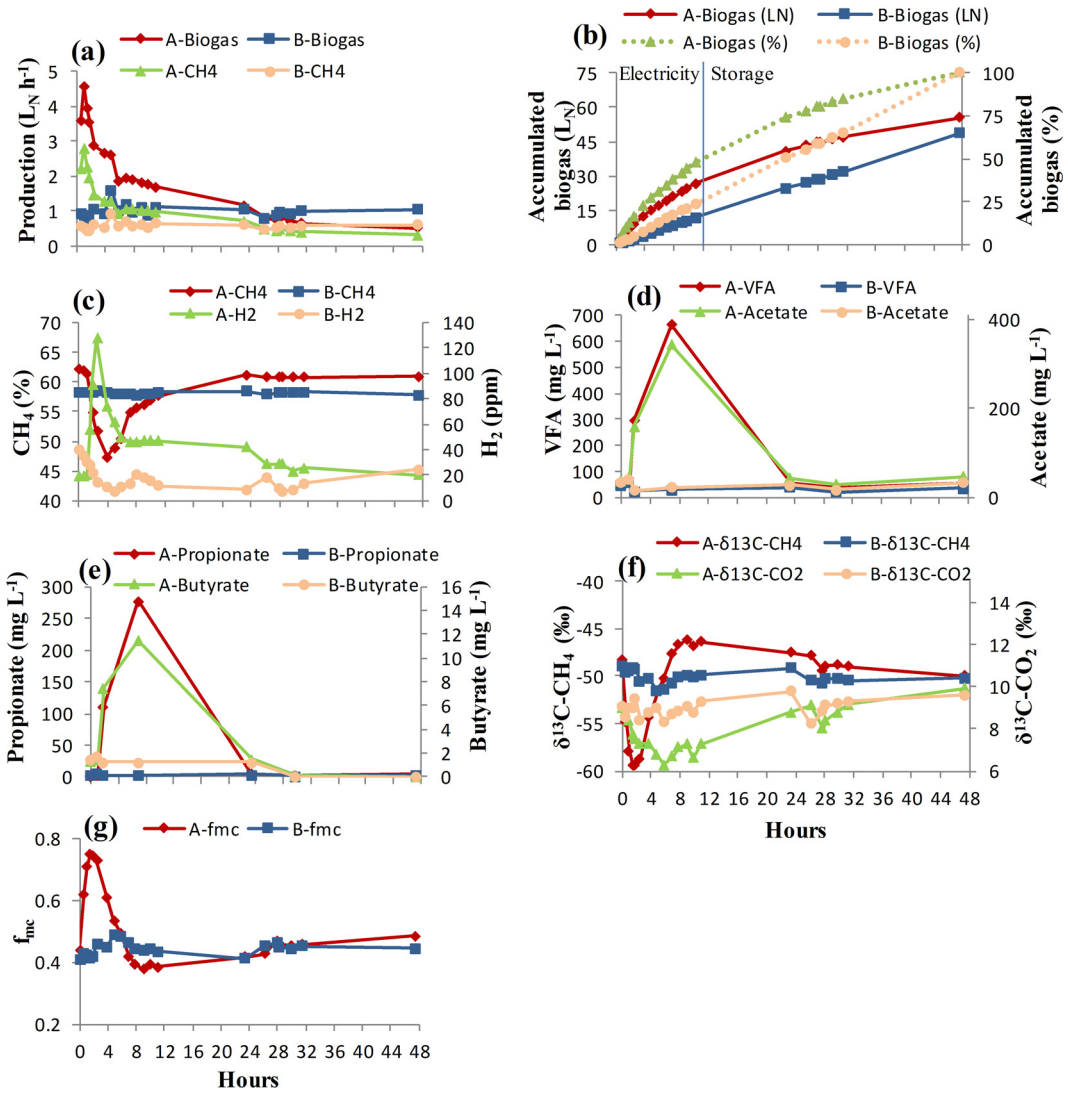


**FIG 2** Short-term process parameters (a to e) and carbon isotope signatures of $CH_4$ and $CO_2$ (f and g) for reactor A fed every second day and reactor B fed every 2 h in phase III. In panel b, electricity demand is assumed for the first 12 h after the feeding, and biogas storage is assumed during the period of low energy demand (13 to 48 h). $L_N$, normalized liters.

pletion indicating the dominance of HM (21). As described in the Materials and Methods section, the isotope signature of $CH_4$ was used to estimate $f_{mc}$. Interestingly, for all feeding regimes, the amount of $\delta^{13}CH_4$ remained almost constant (−48.2 to −51.8‰) throughout all experimental phases. The same is true for $\delta^{13}CO_2$, which remained in a narrow range of 7.6 to 11.4‰ for both reactors. The calculated $f_{mc}$ ranged from 46 to 54% for both reactors when the values measured before feeding are considered, indicating the almost equal contributions of the two pathways to methane production irrespective of the different feeding intervals.

Short-term carbon isotope signatures were also monitored for a period of 48 h during phase III (Fig. 2f and g). The $\delta^{13}CH_4$ levels were more dynamic in reactor A fed every second day, whereas they remained relatively constant in reactor B fed every 2 h. In the reactor fed every second day, $\delta^{13}CH_4$ levels decreased after feeding and reached the minimum value of −59.4‰ at 2 h. The calculated $f_{mc}$ was 0.75 at 2 h, showing that HM dominated after feeding. The subsequent increase in the $\delta^{13}CH_4$ level to the highest value of −46.1‰ at 9 h shows the shift from HM to AM over time ($f_{mc}$, 0.38 at 9 h). During nonfeeding periods between 12 and 48 h, $CH_4$ was produced through both pathways ($f_{mc}$ range, 0.41 to 0.49). The depletion and enrichment of $^{13}C$ in $CH_4$ within 2 h and later on were highly correlated with an increase in $H_2$ and acetate levels during these periods, respectively (Fig. 2c and d). Since $H_2$ is a substrate for HM and acetate is a substrate for AM, the temporal variation in $\delta^{13}CH_4$ levels (hence, methanogenesis) reflects the availability of substrate. The slight depletion in $^{13}C$ of $CO_2$ from 9.0‰ to 6.3‰ at 6 h could reflect the dilution of the bicarbonate pool by the newly produced $CO_2$ through hydrolysis/fermentation steps. These temporal trends in isotope signatures were not observed in reactor B fed every 2 h. Since the $H_2$ and acetate levels remained stable after feeding, the $\delta^{13}CH_4$ and $\delta^{13}CO_2$ levels in the reactor fed every 2 h hardly changed after feeding, indicating the less dynamic nature of the process. The two pathways almost equally contributed to methane production in this reactor ($f_{mc}$ range, 0.41 to 0.49). We have estimated the average amount of

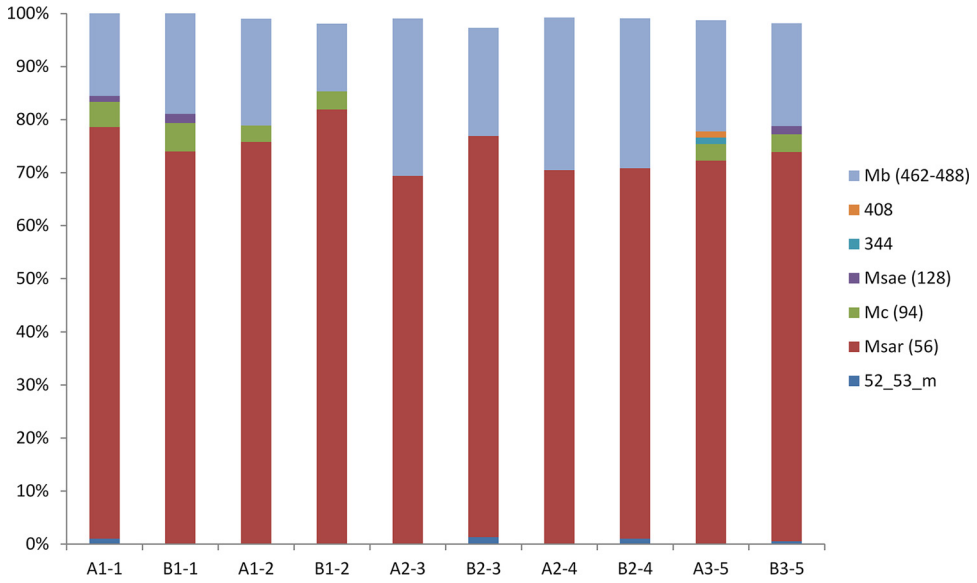


**FIG 3** T-RFLP profiles of *mcrA* and *mrtA* functional genes describing the methanogenic community structure. The sample names consist of numbers and letters: the capital letters (A and B) refer to reactors A and B, the numbers next to the capital letters indicate the phases (1 to 3 indicate phases I to III, respectively), and the numbers after the hyphen represent the sampling time points (numbers 1 to 5 represent samples collected on days 16, 29, 37, 59, and 81, respectively). Mb, *Methanobacterium*; Msae, *Methanosaeta*; Mc, *Methanoculleus*; Msar, *Methanosarcina*. Numbers in the key refer to the size of TRFs (in bases).

methane produced through HM between two feeding events (within 48 h) by multiplying the $f_{mc}$ values (Fig. 2g) and the level of biogas production (Fig. 2a). The result showed that irrespective of the dynamics in methanogenesis in the reactor fed every second day, on average, about 47% of the $CH_4$ was produced through HM in both reactors.

**Microbial community structure.** The community structure of bacteria and methanogenic archaea was studied by use of the T-RFLP profiles of 16S rRNA and the *mcrA* and *mrtA* functional genes, respectively. Community members with a relative abundance of 1% or less were excluded from the T-RFLP analysis. As shown in Fig. 3, the diversity of the methanogens in all reactors was hardly affected by the different feeding regimes, and it can be described as the dominance of the genus *Methanosarcina* (69 to 83%), followed by the genus *Methanobacterium* (11 to 31%). A low abundance of the genus *Methanoculleus* was observed in phases I and III. Members of the genus *Methanosaeta* were detected on a few occasions with a very low abundance. Moreover, minor contributions of other methanogens not matching our database clone sequences were detected by T-RFs.

The resulting bacterial T-RFLP profiles of all reactors were characterized by a large number of distinct peaks and more diverse and varied bacterial groups (see Fig. S2 in the supplemental material). Figures S2a and b in the supplemental material show the T-RF patterns determined with the restriction endonuclease enzymes HaeIII and MspI, respectively. A total of 37 and 43 different T-RFs were observed using the HaeIII and MspI enzymes, respectively. In general, the HaeIII data set yielded a pattern similar to that obtained with the MspI data set.

Further insight into the relationship between the observed bacterial community patterns and the reactor parameters (pH; TAN, total VFA, acetate, propionate, and butyrate concentrations; SBP; VSR; $CH_4$ content and production; $CO_2$ content; $H_2$ concentration; level of biogas production; VOA concentration; and VOA/TIC ratio) was investigated more extensively by multivariate analyses (Fig. 4). The bacterial community structure was investigated from a total of 14 samples (7 samples for each reactor) collected at six different sampling points during phases I to III (two different sampling points for each phase) and one sampling point in phase IV. The community dynamics were similar in the HaeIII and MspI data sets, but the correlation with the reactor parameters was not that strong in the case of the MspI data set. Except for phase I, there was a clear segregation between the bacterial community structures of the two reactors fed under different feeding regimes. The results for samples collected from the two reactors in phase I clustered together, as they were operated under identical conditions. The clear segregation of the results for the two reactors during phases II to IV and the clustering of the results for the two reactors during phase I showed that the bacterial community structure was clearly affected by the change in feeding regimes. Moreover, the community structures for samples from the same feeding regime over time were more similar to each other than those for samples from the same reactor fed with different feeding regimes. A statistical comparison of the bacterial community structure and the reactor parameters suggested that the observed T-RFLP patterns (generated using the HaeIII enzyme) were best explained by the difference in the concentrations of TAN and $H_2$ and pH values (statistically highly significant, $P < 0.01$), as well as by the SBP and concentrations of total VFAs and acetate to some extent (statistically significant, $P < 0.05$). On the other hand, the pH value and the concentrations of $H_2$, TAN, total VFAs, and acetate were the relatively main reactor parameters ($P < 0.05$) explaining the observed bacterial community patterns (generated using the MspI enzyme).

## DISCUSSION

Since the recent introduction of the concept of flexible biogas production, a comprehensive study that looks at the effects of

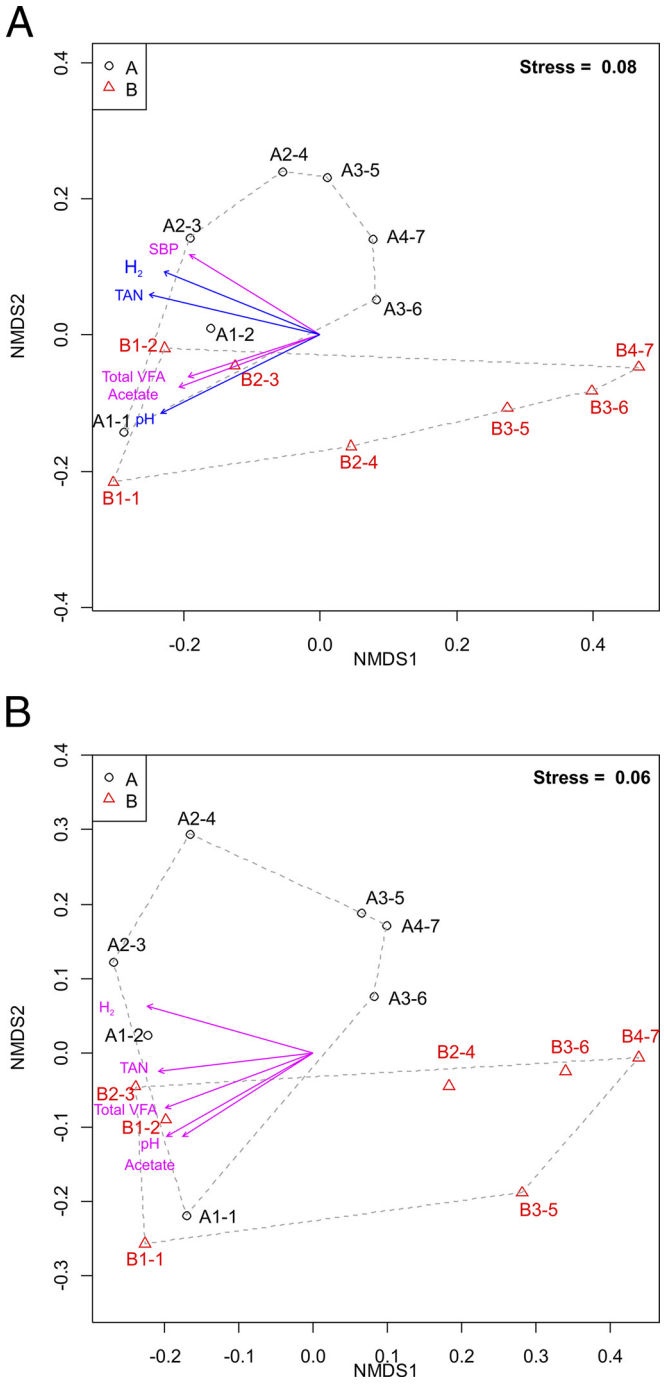


**FIG 4** Nonmetric multidimensional scaling (nMDS) ordination of bacterial community T-RFLP data for 14 samples collected from two DDGS-fed reactors (reactors A and B) over phases I to IV with HaeIII (A) and MspI (B) restriction enzyme digestion. Statistically highly significant ($P < 0.01$) and significant ($P < 0.05$) reactor parameters correlating with the community structures are indicated by blue and magenta solid arrows, respectively. The direction of the arrows shows the correspondence to the community structures, and the length of the arrows indicates the strength of the correlation with the ordination axis. Samples from reactors A and B are indicated with open circles and triangles, respectively, and the digester name is displayed. The sample names consist of numbers and letters: the capital letters (A and B) refer to reactors A and B, the numbers next to the capital letters indicate the phases (1 to 4 indicate phases I to IV, respectively), and the numbers after the hyphen represent the sampling time points (numbers 1 to 7 represent samples collected on days 16, 29, 37, 59, 81, 107, and 115, respectively).

flexible biogas production on process performance, microbial community, and methanogenesis has yet to be published. The present study looked at the possibility of feeding management as a strategy for flexible biogas production in DDGS-fed CSTRs. Moreover, the influences of changing feeding intervals on the biogas production process, microbial community structure, and methanogenesis were also assessed. The results demonstrate that less frequent feeding of larger amounts of substrate at once while keeping the same overall OLR led to a higher biogas production rate immediately after feeding and that feeding could be stopped for at least 2 days during the period of low energy demand. Compared to the conventional more frequent feeding regimes, less frequent feeding regimes achieved flexible biogas production with a higher process efficiency and an improved stability of the process. Moreover, changes in feeding intervals influenced the bacterial community composition, whereas methanogenic communities remained stable.

Our results demonstrate that a change in feeding regimes from more to less frequent feeding while keeping the same overall OLR leads to the production of a larger amount of biogas after feeding and a smaller amount of biogas during the nonfeeding period, indicating the suitability of the latter for flexible biogas production. Our results also showed that $H_2$ and VFAs accumulated as products of the fermentation step after feeding but returned to normal levels during the nonfeeding period. Such a time-dependent dynamic in the amounts of fermentation products was also reflected in the $\delta^{13}CH_4$ values as a result of the temporal variation in methanogenesis. Our results are in agreement with those of an earlier study, where less frequent feeding led to a transient accumulation of VFAs after feeding and a subsequent reduction of the VFA level to normal levels before the next feeding event (9). Mauky et al. also observed short-term increases in the levels of VFA after feeding events, but no accumulation in less frequently fed CSTRs occurred over a long operating period (10), indicating that the long-term stability of the biogas production process was not affected by flexible biogas production.

Another key finding of this study was the improved process efficiency (higher methane and biogas yields of up to 14 and 18%, respectively) and the higher tolerance of the less frequently fed reactor against substrate overloading compared to the process efficiency and tolerance against substrate overloading of the conventional more frequently fed reactor. Our results are in agreement with those of a previous study where a less frequently fed reactor appeared to have a higher degree of functional stability and even more tolerance to an organic shock load (12). The improved process performance in the less frequently fed reactor was not due to experimental and measurement errors since the initial reactor conditions were almost identical and the gas meters were working properly. Instead, two possible explanations for why the process efficiency differed with different feeding intervals can be provided. Since VFAs did not accumulate over a long operating period in either of the reactors, the difference in methane yields was governed by the first two stages of anaerobic digestion (the hydrolysis and acidogenesis steps). One possibility is the difference in the average time that the freshly added substrate spends in a reactor before effluent is taken from the reactor. For reactors fed daily, every second day, and every 2 h, effluents were taken out once per day and at 2-day and 2-h intervals, respectively. This means that for substrate containing both slowly and rapidly degradable fractions, there was a high probability that the slowly

degradable fraction of the substrate would be partially removed before degradation was completed in the reactor fed every 2 h, whereas the slowly degradable fraction had more time before it was taken out of the reactors fed daily and every second day, which could give a considerable time for the substrate to be degraded further. This is a possible explanation based on the fact that the chemical compositions of particulate substrates, such as DDGS, maize silage, and other solid agricultural waste materials, are generally heterogeneous and such substrates contain rapidly degradable as well as slowly degradable fractions (28–30). Nevertheless, further work is needed to determine the rate of hydrolysis of DDGS in batch reactors and the composition of rapidly and slowly degradable fractions.

The second explanation is that a change in the feeding interval could have led to a different environment that was conducive to the growth and activity of bacteria. Our results demonstrate that there was a change in the bacterial community, TAN concentration, and VSR with the different feeding regimes. Moreover, the less frequently fed reactor showed a highly dynamic environment associated with a transient accumulation of fermentation products (VFAs, $H_2/CO_2$) and a temporal variation in pH compared to the characteristics of the less dynamic reactor fed every 2 h. Such a dynamic environment was also confirmed by the short-term isotope measurements, showing a temporal variation in methanogenesis pathways. The changes in bacterial community structure under the three feeding regimes were best correlated with the differences in the concentrations of TAN and $H_2$ and pH values, as indicated by multivariate analysis of the T-RFLP profiles. The dynamic environment within the less frequently fed reactor could provide more functional niches for the hydrolyzing and acidogenic bacteria to grow and degrade the DDGS efficiently. Different pH values, redox potentials, and concentrations of fermentation products have been shown to affect the growth and activity of fermenting microorganisms (30). The higher TAN concentration in the less frequently fed reactor than in the more frequently fed reactor also indicates a more thorough degradation of DDGS since TAN is a product of protein fermentation. Moreover, a higher VSR was observed in this reactor, also indicating the improved degradation of DDGS in the less frequently fed reactor. Nevertheless, the observed higher biogas and methane yields in the less frequently fed DDGS-fed reactor cannot be generalized to reactors run with other substrates. Since different substrates have different proportions of slowly and easily degradable fractions, further research is warranted to improve our understanding of the process efficiency of reactors fed with different substrates and at different feeding intervals.

The bacterial community composition varied under different feeding regimes, whereas methanogens remained stable in our DDGS-fed reactors. Our results are in agreement with the findings of previous studies where the methanogenic community composition remained stable in the reactors fed daily and every second day, whereas the bacterial community differed substantially, with higher levels of dynamics in the latter (12). Moreover, a higher diversity of the bacterial community than the methanogen community was observed irrespective of the feeding intervals. This was not surprising, as bacteria are involved in several steps of biomass degradation from hydrolysis to acetogenesis, whereas methanogens are responsible only for the production of methane. The absence or low abundance of *Methanosaeta* but the dominance of *Methanosarcina* in all the DDGS-fed reactors, despite the difference in feeding regimes, might be due to the relatively high TAN levels in these reactors (2.6 to 3.6 g $NH_4^+$-N liter$^{-1}$) (31–33). It is well-known that *Methanosarcina* and hydrogenotrophic methanogens are more tolerant of higher ammonia levels (33), whereas members of the genus *Methanosaeta* are sensitive to ammonia (33) and may no longer be detected at TAN concentrations exceeding 2.5 g $NH_4^+$-N liter$^{-1}$ (26, 34).

Similar to our findings, it was previously shown that *Methanosarcina* dominated DDGS-fed reactors operated at a high OLR of 5 g VS liter$^{-1}$ day$^{-1}$ with a TAN concentration of 2.94 g $NH_4^+$-N liter$^{-1}$, whereas *Methanosaeta* dominated at a low OLR of 2 g VS liter$^{-1}$ day$^{-1}$ with a TAN concentration of 1.82 g $NH_4^+$-N liter$^{-1}$ (18). A high TAN level has previously been shown to inhibit AM in favor of methane production through SAO coupled to HM (31, 32). We do not have direct evidence for SAO in our DDGS-fed reactors, but previous studies observed relatively high levels of *Methanosarcina* in SAO-dominated processes (33, 35–37). Since SAO is endergonic under standard conditions, the hydrogenotrophic *Methanobacterium* organisms might be involved in the reduction of the $H_2$ partial pressure to a level for SAO to be energetically favorable. Syntrophic acetate-oxidizing bacteria (SAOB) capable of SAO were detected in laboratory-scale mesophilic CSTRs with high ammonia concentrations (37) and in industrial mesophilic or thermophilic CSTRs (38). Another recent finding has also shown that high levels of ammonia in mesophilic swine waste digesters induce a shift from AM to SAO and the latter is carried out by a dynamic and heterogeneous community with this metabolic capacity rather than known defined cultures of SAOB (39). Further research on the identity of the bacterial communities in our DDGS-fed reactors would provide a better insight into the role of each functional group within the bacterial community.

The practical implication of this study is that by feeding less frequently, more biogas can be produced for hours after the feeding event to meet a high energy demand and during a period of low energy demand feeding can be stopped for at least 2 days. In the reactor fed every second day, almost 50% of the total biogas produced from a single feeding event was generated within the first 12 h, whereas only 25% of the biogas was generated during the same time period in the reactor fed every 2 h (Fig. 2b). If electricity demand within the first 12 h after the feeding of the reactor fed every second day is assumed and no energy demand between 13 and 48 h is assumed, then 50% and 75% of the biogas generated after 12 h by the reactors fed every second day and 2 h, respectively, must be stored intermittently. This simplified calculation demonstrates that longer feeding intervals (e.g., feeding every second day) could allow either a more flexible electricity production capability or a lower demand for storage capacity, which would thus provide savings by avoiding the need to make extra investments in storage capacity. More rigorous calculations and different scenarios need to be considered for practical implementation (10). From a practical standpoint, less frequent feeding could provide easier plant operation and mechanical stability due to the possibility of using manual and less frequent feeding but warrants further research about its advantage in full-scale biogas plants.

In conclusion, this study demonstrates that flexible biogas production that adapts biogas output to energy demand can be regulated by feeding less frequently without affecting the long-term stability of the process. In addition to flexibility, significantly higher methane yields and an improved stability of the process against organic overloading were achieved by feeding less fre-

quently. The methanogenic community remained stable under different feeding regimes, while the temporal variation in the isotopic composition of methane reflects the change in the activity of methanogens in the reactors fed less frequently. The bacterial community composition varied between reactors fed at different intervals, and these changes were strongly correlated with the difference in some of the process parameters. Nevertheless, further research is needed to demonstrate the benefit of a less frequent feeding regime in large-scale reactors that use DDGS and also reactors that use other substrates.

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